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Review

Adenosine receptor containing oligomers: Their role in the control of dopamine and glutamate neurotransmission in the brain ☆

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ABSTRACT

While the G protein-coupled receptor (GPCR) oligomerization has been questioned during the last fifteen years, the existence of a multi-receptor complex involving direct receptor–receptor interactions, called receptor oligomers, begins to be widely accepted. Eventually, it has been postulated that oligomers constitute a distinct functional form of the GPCRs with essential receptorial features. Also, it has been proven, under certain circumstances, that the GPCR oligomerization phenomenon is crucial for the receptor biosynthesis, maturation, trafficking, plasma membrane diffusion, and pharmacology and signalling. Adenosine receptors are GPCRs that mediate the physiological functions of adenosine and indeed these receptors do also oligomerize. Accordingly, adenosine receptor oligomers may improve the molecular mechanism by which extracellular adenosine signals are transferred to the G proteins in the process of receptor transduction. Importantly, these adenosine receptor-containing oligomers may allow not only the control of the adenosinergic function but also the fine-tuning modulation of other neurotransmitter systems (i.e. dopaminergic and glutamatergic transmission). Overall, we underscore here recent significant developments based on adenosine receptor oligomerization that are essential for acquiring a better understanding of neurotransmission in the central nervous system under normal and pathological conditions. This article is part of a Special Issue entitled: "Adenosine Receptors".

Contents

1. Introduction

Adenosine is an endogenous nucleoside mostly formed as a degradation product of adenosine triphosphate (ATP) and to a lesser degree from *S*-adenosyl-L-homocysteine (SAH) metabolism. As soon

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as adenosine has been generated it can be either intracellularly phosphorylated to form AMP or eliminated out of the cells by means of ubiquitous nitrobenzylthioinosine-sensitive equilibrative nucleoside transporters (ENTs). Additionally, adenosine can react with L-homocysteine to form SAH. Finally, both intra- and extracellular adenosine can be deaminated to form inosine by the action of intra- and ecto-adenosine deaminase, respectively. Interestingly, as an intermediate metabolite in several biochemical pathways, adenosine plays a key regulatory role in numerous physiologic processes, including platelet function, coronary and systemic vascular tone, and lipolysis in adipocytes. In the nervous system, since the extracellular concentration of adenosine raises as a function of the neuronal activity, it acts as an energy dependent neuromodulator through combined presynaptic, postsynaptic and non-synaptic actions [1,2]. Therefore, extracellular adenosine regulates several functions in the brain, including neuronal viability, neuronal membrane potential, propagation of action potentials, astrocytic functions, microglia reactivity, primary metabolism in both neurons and astrocytes, and blood flow [2]. Consequently, the physiological roles and potential therapeutic use of adenosine have been extensively revised over the last years [3,4].

Adenosine mediates its actions through the activation of specific GPCRs, for which four subtypes have been identified (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) [5]. These receptors have a distinctive pharmacological profile, tissue distribution and effector coupling [6], and its functioning has been extensively studied in the central nervous system (CNS) (Table 1). Interestingly, A_1Rs and $A_{2A}Rs$ are largely responsible for the central effects of adenosine [7]. The most abundant and homogeneously distributed adenosine receptor in the brain is the inhibitory A_1R, which is functionally coupled to members of the pertussis toxin-sensitive family of G proteins ($G_{i/o}$) and whose activation regulates the activity of membrane and intracellular proteins such as adenylate cyclase, Ca^{2+} channels, K^+ channels and phospholipase C (Table 1) [8]. In contrast, the $A_{2A}R$ is expressed at high levels in only a few regions of the brain, namely the striatum, the olfactory tubercle and the nucleus accumbens [9]. The $A_{2A}R$ is mostly coupled to G_s in the peripheral systems but mediates its effects predominantly through activation of G_{olf} in the striatum [10], thus activating adenylyl cyclase which in turn converts ATP into cAMP (Table 1). Next, the $A_{2B}R$ is positively coupled to adenylyl cyclase and PLC through a G_s and G_q protein, respectively [11] (Table 1). The $A_{2B}R$ is thought to be fairly ubiquitous in the brain, but the association of the $A_{2B}R$ to specific physiological or behavioral responses remains quite difficult because of the lack of good $A_{2B}R$ selective agonists or antagonists [12]. Ultimately, the A_3R has also been found to inhibit adenylyl cyclase, and to promote calcium mobilization probably by direct G protein βγ-subunits PLC activation (for review see [3]) (Table 1). Interestingly, in humans the A_3R is a high affinity receptor antagonized by xanthines [13,14]. Finally, it is important to mention here that different classes of proteins, other than G proteins, have been shown to interact with adenosine receptors and to eventually modify their functioning [15].

2. Adenosine receptor containing oligomers

The concept of GPCRs oligomerization was first postulated in the 1980s by Luigi F. Agnati and Kjell Fuxe, who proposed that an intramembrane neuropeptide and monoamine receptor–receptor interaction was responsible for the functional cross-talk observed between these two neurotransmitter systems [16,17]. The initial indirect evidence pointing to the existence of GPCR oligomers containing neuropeptide and monoamine receptors was established by means of radioligand-binding experiments. Thus, by using this approach the existence of interactions between neurokinin NK1 and 5-HT_1 receptors, CCK-2 and serotonin 5-HT_2 receptors, vasoactive intestinal peptide (VIP) and serotonin 5-HT_1 receptors, cholecystokinin CCK-2 and dopamine D_2 receptors, neurotensin NTS1 and dopamine D_2 receptors, and neuropeptide Y (NPY) and α_2 adrenergic receptors were established (for review see [18]). In addition, some photo-affinity labelling and radiation inactivation experiments, together with hydrodynamic and cross-linking analysis, also supported the GPCR oligomerization idea [19–22]. Later on, as the existence of some GPCR homodimers in native tissue was demonstrated (i.e. the dopamine D_2 and the adenosine A_1 receptor homodimers in the brain [23,24]), the GPCR oligomerization issue began to gain significance, given the potential physiological implications of this phenomenon. Nevertheless, the straight evidence for a direct receptor–receptor interaction within the GPCR oligomerization phenomenon was achieved by means of biophysical techniques based on resonance energy transfer (RET), like bioluminescence-RET (BRET) and fluorescence-RET (FRET) (for review see [25]). In addition, the combination of some fluorescence-based approaches allowed the identification of higher-order GPCR oligomers or receptor mosaics (RMs) in living cells [26–32]. Finally, these powerful approaches have been recently used to demonstrate the existence of GPCR oligomers in

Table 1
Adenosine receptors.

Receptor	Adenosine affinity (EC_{50})[a]	G protein	Transduction mechanisms[b]	Physiological actions in brain
A_1R	~50 nM	$G_{i1,2,3}$[c]	Inhibits AC (↓cAMP) Activates PLC (↑IP_3/DAG) Activates PLA2 (↑AA) Activates PLD (↑PEtOH) Activates GIRKs Inhibits Ca^{2+} channels	Inhibits synaptic transmission; hyperpolarizes neurons.
		G_o		
$A_{2A}R$	~1 μM	G_S [c]	Activates AC (↑cAMP)	Facilitates transmitter release; regulation of sensorimotor integration in basal ganglia.
		G_{olf}	Activates AC (↑cAMP) ↑IP_3 Inhibits Ca^{2+} channels	
		$G_{15,16}$ [d]		
$A_{2B}R$	>10 μM	G_S [c]	Activates AC (↑cAMP) Activates Ca^{2+} channels	Increases in cAMP in brain slices
		$G_{q/11}$ [d]	Activates PLC (↑IP_3/DAG)	
A_3R	~50 nM	$G_{i2,3}$[c]	Inhibits AC (↓cAMP) Activates PLC (↑IP_3/DAG)	Uncouples A_1R and mGlu receptors

[a] Data are from a cyclic AMP functional assay in CHO cells expressing the corresponding adenosine receptor [55]. The assay is performed in the presence of the adenosine transport inhibitor nitrobenzylthioinosine (NBTI) to avoid adenosine uptake.
[b] AC, adenylyl cyclase; PLC, phospholipase C; IP_3, inositol triphosphate; DAG, diacylglycerol; PLA2, phospholipase A2; PEtOH, phosphatidylethanol; GIRKs, G protein-dependent inwardly rectifying K^+ channels; AA, arachidonic acid.
[c] Main mechanism of coupling.
[d] Receptor transfected cell system.

native tissue (e.g. the presence of oxytocin receptor dimers and/or oligomers in mammary glands) [33].

In the last years, a set of publications have demonstrated the existence of adenosine receptors containing oligomers. For instance, the A_1R has been found to heteromerize with the dopamine D_1 receptor (D_1R), this phenomenon being essential for differential desensitization mechanisms and for receptor trafficking [34]. Similarly, the heterodimer composed by the A_1R and the metabotropic glutamate type 1α ($mGlu_{1\alpha}$) receptor seems to play a key role in preventing glutamate excitotoxicity [35], whereas the physiological role for the proved heteromerization of the A_1R and the purinergic $P2Y_1$ receptor ($P2Y_1R$) is still under debate [36]. On the other hand, the $A_{2A}R$ also possesses the ability to oligomerize with the dopamine D_2 receptor (D_2R) [37,38]. Interestingly, the latter receptor–receptor interaction underlies the molecular mechanism behind the antagonistic adenosine-dopamine interactions that regulate the function of the GABAergic enkephalinergic neurons [39,40]. Finally, the $A_{2A}R$ also heteromerizes with the metabotropic glutamate type 5 ($mGlu_5$) receptor [41], and in this case, a synergistic functional interaction has been demonstrated at both biochemical and behavioral levels [42–44].

2.1. The adenosine A_1 and A_{2A} receptors heterodimer ($A_1R/A_{2A}R$) and the control of glutamate release in the central nervous system

A_1Rs and $A_{2A}Rs$ operate through opposing signal transduction pathways (Table 1). Thus, they have been historically considered as independent entities in the framework of adenosine physiology. Interestingly, these adenosine receptors were shown to form homodimers in both ectopic expression systems and in native tissue [24,45]. It was then postulated that the functional receptor placed at the cell surface of cells was represented by a homodimer [45]. In addition, it was shown that A_1Rs and $A_{2A}Rs$ were coexpressed in a variety of cells like glutamatergic neurons of the hippocampus [46] and the striatum [47]. Furthermore, heterodimers involving A_1Rs and $A_{2A}Rs$ were described in the context of the modulation of the glutamatergic neurotransmission in the central nervous system [47]. Nevertheless, the existence of these dimeric forms involving either A_1Rs or $A_{2A}Rs$ did not first alter the widespread acceptance that these two adenosine receptors with opposite physiological functions would signal independently. However, some evidence strongly indicated a potential interaction between A_1Rs and $A_{2A}Rs$ [48–50][48], suggesting the possibility that these receptors could establish a molecular and/or functional cross-talk, i.e. as if they were part of the same molecular transduction complex or signalosome, thus affording a logical and economical device to fine-tune adenosine neuromodulation.

Initially, as described for the neuropeptide and monoamine receptor–receptor interaction studies (see above), the functional cross-talk between the A_1R and the $A_{2A}R$ was assessed by means of radioligand-binding experiments. Thus, these approaches revealed that the formation of the $A_1R/A_{2A}R$ heteromer *per se* did not modify the affinity of A_1Rs or $A_{2A}Rs$ for their respective agonists. But interestingly, when the $A_1R/A_{2A}R$ heteromer was challenged with an $A_{2A}R$ agonist, the affinity of the A_1R for its agonist decreased significantly. Conversely, the activation of the $A_1R/A_{2A}R$ heteromer with an A_1R agonist did not alter the $A_{2A}R$ binding characteristics. Therefore, it could be concluded that, within the $A_1R/A_{2A}R$ heteromer, the agonist-mediated A_1R responsiveness is modulated by the $A_{2A}R$, whereas the binding properties of the latter were not altered by the A_1R challenge. This ability of the $A_{2A}R$ to control the A_1R within the $A_1R/A_{2A}R$ heteromer has been further confirmed by functional experiments (i.e. determination of receptor-mediated second messenger generation). For instance, upon the formation of the $A_1R/A_{2A}R$ heteromer, the $A_{2A}R$ challenge significantly decreased the A_1R-mediated intracellular calcium mobilization [47]. Overall, these results suggest that under certain circumstances the $A_{2A}R$ can control the A_1R functionality, whereas the reverse does not happen.

The question that is still remaining focuses on whether the formation of the $A_1R/A_{2A}R$ heteromer might play a physiological role in native brain preparations. This issue is of major relevance, since the involvement of GPCR oligomerization in pathophysiological conditions remains unexplored. Interestingly, there is strong evidence pointing to the existence of the $A_1R/A_{2A}R$ heteromer in the brain areas where the distribution of A_1Rs and $A_{2A}Rs$ clearly overlaps, for instance in the dopamine-rich regions [51,52]. Indeed, ultrastructural analysis by immunolelectron microscopy experiments in the rat striatum have revealed that these receptors codistribute and colocalize in striatal glutamatergic synapses [47] (Fig. 2). In addition, co-immunoprecipitation studies have clearly shown that the A_1R and $A_{2A}R$ are co-purified from rat striatal synaptosomes when specific antibodies are used, thus suggesting the existence of $A_1R/A_{2A}R$ heteromers in the striatal glutamatergic synapses and/or perisynaptic regions [47].

In the excitatory glutamatergic synapses the most accepted role for adenosine is the inhibition of synaptic transmission (i.e. inhibition of glutamate release) through the activation of presynaptic A_1Rs. In fact, the A_1R constitutes the prototypical inhibitory $G_{i/o}$ protein-coupled receptor whose stimulation decreases the probability of neurotransmitter release [53]. On the other hand, the use of particular stimulating conditions together with pharmacological tools with adequate selectivity to hamper $A_{2A}R$ functioning, has made possible the identification of a parallel capacity of adenosine to facilitate the

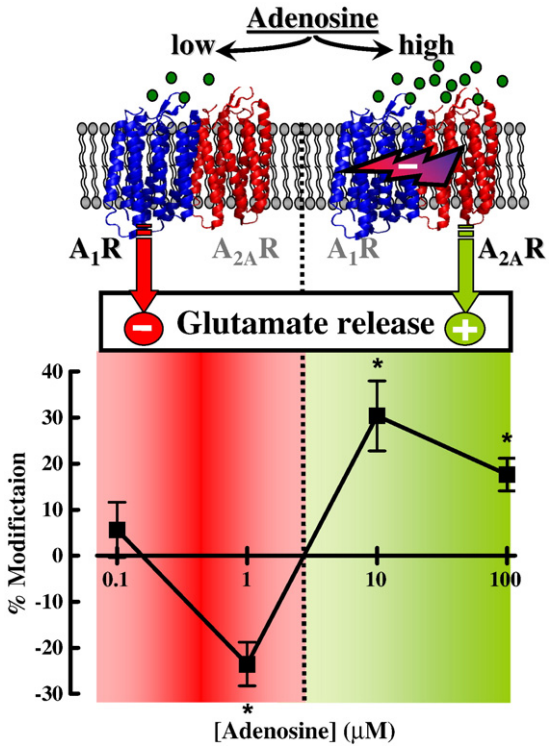

Fig. 1. Scheme of the proposed concentration-dependent adenosine receptor heterodimer switch. Upper panel shows the efficacy of adenosine to stimulate A_1Rs and $A_{2A}Rs$ within the $A_1R/A_{2A}R$ heterodimer. Low concentrations of adenosine activate predominantly A_1Rs, which inhibits glutamate release. High concentrations of adenosine also activate $A_{2A}Rs$, which, by means of the $A_1R/A_{2A}R$ intramembrane interaction (blue-red arrow), antagonizes A_1R function, therefore facilitating glutamate release. The schematic GPCRs diagrams were prepared using PyMOL (PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, USA), with the crystal structure of the sensory rhodopsin II (PDB: 1JGJ) as models. In the lower panel the proposed kinetics of the $A_1R/A_{2A}R$ heterodimer-mediated glutamate release control is shown. Thus, increasing concentrations of adenosine (0.1–100 μM) produced a biphasic effect, with low and high concentrations inhibiting and stimulating the evoked release of glutamate in striatal synaptosomes, respectively.
Adapted from Ref. [47]

evoked glutamate release via activation of presynaptic A_{2A}Rs [54] (Fig. 2). Therefore, one can easily ask the following question: how does adenosine decide between A_1Rs and A_{2A}Rs in the glutamatergic nerve terminal in order to control glutamate release? To answer this question several facts need to be primarily established. First, that the A_1R and the A_{2A}R have different affinities for adenosine with the former showing a higher affinity for this nucleoside than the latter (Table 1) [55]. Therefore, at low concentrations of adenosine the A_1R will be primarily stimulated and glutamate release inhibited (Fig. 1). In contrast, at high concentrations of adenosine both A_1Rs and A_{2A}Rs could potentially be activated, and thus the question arises how the control of glutamate release will be established under these circumstances. How can the integration of A_1Rs and A_{2A}Rs signals take place? The evidence described so far point to the fact that the A_1R/A_{2A}R heteromer located at the glutamatergic terminal makes possible this integration, since the A_1R responsiveness within the heteromer is under the control of the A_{2A}R. Therefore, at high concentrations of adenosine the A_{2A}R stimulation will abrogate the A_1R functioning by means of a phenomenon of allosteric cross-inhibition, which will lead to predominant facilitation of glutamate release (Fig. 1). Then, it can be concluded that A_1R/A_{2A}R heteromerization provides a molecular framework that allows adenosine to exert a fine-tune modulation of glutamate release in the striatum as the A_1R/A_{2A}R heteromer is found in the glutamatergic terminals of the striatal spine module (SSM) (see below and Fig. 2). Thus, the extracellular adenosine switches from inhibition to facilitation thanks to the A_1R/A_{2A}R heteromer [47]. It is important to keep in mind that this scenario might be true in brain and related to the control of neurotransmitter release, where a proper A_1R and A_{2A}R co-localization and stoichiometry allow the existence of a balanced A_1R/A_{2A}R oligomer. However, in the situation where either an unbalanced A_1R/A_{2A}R oligomer exist (i.e. existence of spare A_{2A}R that sensitize the A_{2A}R-mediated adenosine response) or that the A_1R and the A_{2A}R do not oligomerize (i.e. do not co-distribute), then the predominance of a distinctive adenosine receptor-mediated function (A_1R or A_{2A}R) will be observed, as it has been recently described [56]. Thus, more work is needed to fully characterize the functionality of the A_1R/A_{2A}R oligomer outside the SSM and also the mechanism by which the oligomer couples to G protein(s) (i.e. do the A_1R/A_{2A}R oligomer couples to a single G protein or simultaneously to G_i and G_S?). Overall, the A_1R/A_{2A}R heteromer confers a rationale for the existence of heteromers of isoreceptors (receptors for the same neurotransmitter) and demonstrates that neurotransmitter heteromers composed of isoreceptors with different affinities for their endogenous neurotransmitter can act as concentration-dependent processors that exert a fine-tune modulation of neurotransmission (Fig. 1).

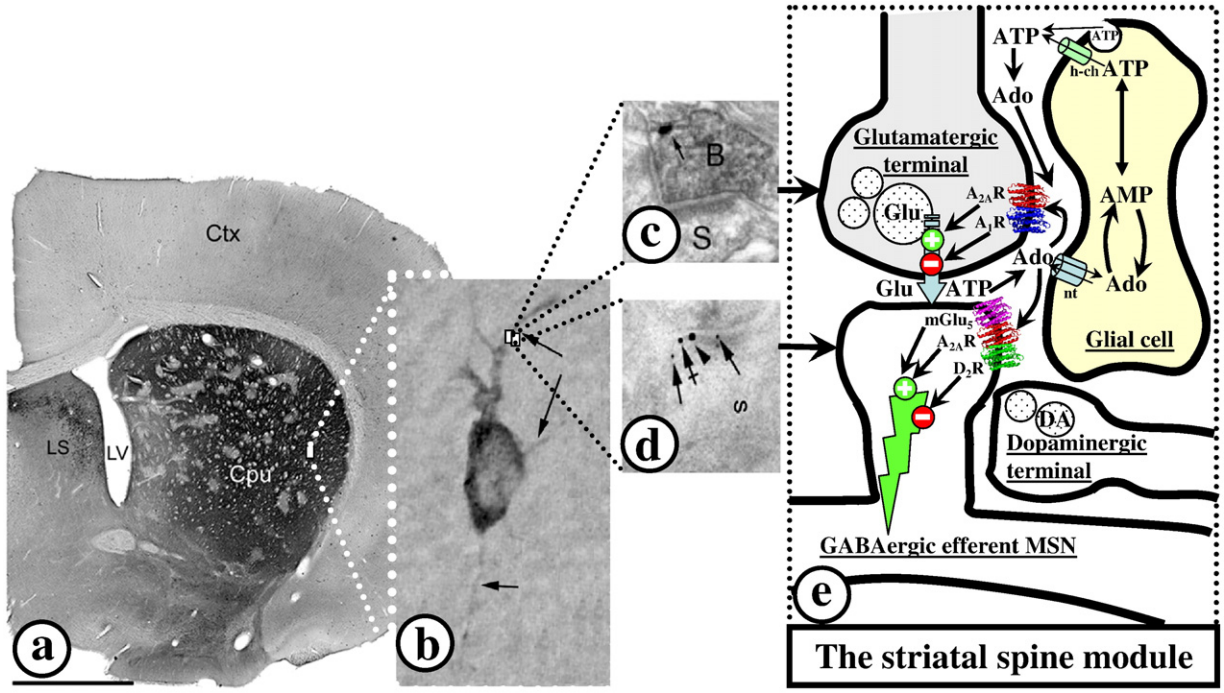

Fig. 2. Schematic representation of the striatal spine module (SSM) within the striatal spiny neuron (MSN). (a) Immunoreactivity of NECAB2, an A_{2A}R interacting protein, in the rat striatum. As described for the A_{2A}R the immunoreactivity was very strong in the caudate putamen (Cpu) [130] as revealed by a pre-embedding immunoperoxidase method at the light microscopic level. Ctx, cortex; LS, lateral septum; LV, lateral ventricle. Adapted from Ref. [130]. (b) High magnification picture showing immunoreactivity for NECAB2 in a GABAergic neuron of the caudate putamen. Adapted from Ref. [130]. (c) Electron micrograph showing the presynaptic co-localization of A_1Rs and A_{2A}Rs in rat striatum. Peroxidase reaction product (immunoreactivity for A_1Rs) filled axon terminals -B- establishing asymmetrical synapses with spines -S-, in which immunoparticles (immunoreactivity for A_{2A}Rs) were localized along the extrasynaptic plasma membrane (arrows). Adapted from Ref. [47]. (d) Subcellular distribution of A_{2A}R, D_2R and $mGlu_5$ receptor in rat striatum. Electron micrograph showing immunoreactivity for A_{2A}R, D_2R and $mGlu_5$ receptor in rat striatum as revealed using a triple-labeling post-embedding immunogold technique. Immunoparticles for A_{2A}R (10 nm size, arrows), D_2R (15 nm size, crossed arrows) and $mGlu_5$ receptor (20 nm size, arrowheads) were detected along the extrasynaptic and perisynaptic plasma membrane of the same dendritic spine (s) establishing excitatory synaptic contact with axon terminals. Adapted from Ref. [129]. (e) Schematic representation of the SSM. The MSN is the most common neuron in the striatum and it receives two main inputs: glutamatergic afferents from cortical, limbic and thalamic areas, and dopaminergic afferents from the mesencephalon. The A_1R/A_{2A}R heterodimer is located in the glutamatergic terminal and controls the glutamate release. The A_{2A}R/D_2R/$mGlu_5$ receptor oligomer is located in the dendritic spine of the enkephalin medium spiny neuron and controls the excitability of the MSN. The differential stimulation of the units of the receptor heteromers (e.g. A_1R/A_{2A}R and A_{2A}R/D_2R/$mGlu_5$ receptor) determines the predominant signalling pathway (see [42,43]). The extracellular adenosine levels are thought to be regulated by a glial-based adenosine cycle [131]. Glial cells can release ATP via direct release through hemichannels (h-ch) and/or by vesicular release. Also, ATP can be co-released with glutamate at the synaptic cleft. The ATP at the extracellular level is quickly degraded into adenosine (Ado) by ectonucleotidases. Ado can also be released directly via equilibrative nucleoside transporters (nt). At the intracellular level adenosine concentration is mainly controlled by adenosine kinase, which is part of a substrate cycle between adenosine and AMP and finally ATP [131].

3. A_1R containing oligomers

3.1. A_1R and dopamine D_1 receptor (D_1R) oligomers

Dopamine plays a key role in a large variety of functions in the CNS including short-term memory and motor, attention and reward control [57–59]. Imbalance in dopaminergic neurotransmission has been associated with drug addiction [60] and CNS alterations in stress conditions [61]. Furthermore, dysfunction of dopaminergic neurotransmission has been also involved in psychiatric and neurological disorders including Parkinson's disease, schizophrenia and bipolar disorders [62,63]. Dopamine specifically acts on GPCRs located in the plasma membrane of cells and, based on morphological and pharmacological characteristics, these receptors are classified as D_1-like, which include the D_1R and the D_5R, and D_2-like receptors, which include the long and short isoforms of the D_2R (termed $D_{2L}R$ and $D_{2S}R$, respectively), the D_3R and the D_4R [63].

The modulatory effects of adenosine on dopamine systems have been investigated in view of their relevance to human pathology such as schizophrenia and Parkinson's disease. Antagonistic adenosine/dopamine interactions have been widely reported, showing that adenosine can inhibit several effects of dopamine in the cerebral cortex and basal ganglia. In rat models of Parkinson's disease, for instance, it was shown that nonselective adenosine receptor antagonists (i.e. either caffeine or theophyllamine) enhanced the effect of L-DOPA and other dopamine receptor agonists increasing motor activity [64]. Similarly, it was also described in unilaterally 6-OH-dopamine (which specifically destroys the nigrostriatal dopamine pathway) lesioned rats that the A_1R selective antagonist CPT enhanced the motor activating effects of the D_1R selective agonist SKF 38393 [65], while the A_1R selective agonist CPA counteracted SKF 38393-induced grooming behavior [66]. Hence, in order to explain the results obtained in a large number of behavioral studies, it was suggested that antagonistic adenosine/dopamine interactions were, at least in part, caused by an intramembrane interaction between specific subtypes of dopamine and adenosine receptors, in this case A_1Rs and D_1Rs [67,68].

The first evidence of a receptor–receptor interaction involving the A_1R and the D_1R was provided by means of co-immunoprecipitation experiments and confocal microscopy analysis in co-transfected fibroblast Ltk-cells and cortical neurons in culture [69]. Interestingly, this selective A_1R/D_1R heteromerization disappeared after pretreatment with a selective D_1R agonist, but not after combined pretreatment with D_1R and A_1R agonists. Likewise, the D_1R uncoupling from adenylyl cyclase only occurred when the A_1R/D_1R heteromer was co-activated, while the A_1R agonist alone did not substantially reduce the D_1R agonist-induced accumulation of cAMP [69]. Similarly, by means of double immunofluorescence experiments with confocal microscopy, a high degree of A_1R and D_1R co-localization was found in both co-transfected fibroblast cells and cortical neurons in culture [70]. In this study A_1R/D_1R coimmunoprecipitation was also demonstrated in native tissue, for instance in samples from rat nucleus accumbens. Interestingly, in the native tissue A_1R/D_1R coimmunoprecipitation was reduced only when rats were treated with cocaine, while co-activation of A_1Rs prevented receptor uncoupling [70].

The above-commented results may then explain the well-documented antagonistic A_1R/D_1R receptor–receptor interactions found in the neuronal networks of the brain, since they indicate that A_1R/D_1R heteromerization could determine changes in the pharmacological profile of the receptors, for instance, activation of the A_1R could modulate D_1R signaling. One of the mechanisms that may explain this action is based on the ability of A_1R activation within the heteromer to change the binding characteristics of dopamine to the D_1R. Accordingly, it was firstly described in crude membrane preparations from cell lines and rat striatum that activation of A_1Rs reduced the proportion of D_1Rs in the high-affinity state without changing the dissociation constants of the high- and the low affinity binding sites [68,71,72]. Thus, the high affinity form of the receptor would be partially or completely lost in the presence of agonists activating A_1Rs, while in the absence of cross-modulation the binding of dopamine to the D_1R would involve both the low affinity (80-90%) and the high affinity form of the receptor [72]. Similarly, it was reported that the adenosine A_1R selective antagonist DPCPX had an effect on the K_i value of the D_1R selective agonist SKF 38393 in A_1R/D_1R transfected cells, thus adenosine would exert a tonic inhibition of dopamine in the D_1R mediated function through the A_1R/D_1R complex [73]. In addition, radioligand binding experiments performed in cotransfected A_1R/D_1R cells showed that the adenosine A_1R-mediated modulation of D_1R induced a concentration-dependent decrease in the affinity of the D_1R agonist SKF38393 [74]. Overall, it is now well accepted that the simultaneous activation of the A_1R/D_1R heteromer may allow the antagonistic intramembrane receptor–receptor interaction to take place, and it has been suggested that one functional meaning of this interaction is the uncoupling of the D_1R from Gs protein with the disappearance of the D_1R high affinity state [75].

On the other hand, the uncoupling from the downstream signaling effectors may also be dependent of A_1R/D_1R heteromerization, which in fact appears to control the temporal dynamics of receptor activation and down-regulation [69]. As described earlier, upon co-activation of A_1Rs and D_1Rs in co-transfected fibroblast cells and in cortical neurons in culture the heteromer complex was maintained, while during individual treatments heteromerization disappeared, thus indicating that co-activation induced heteromer stabilization. Similarly, pretreatment with an A_1R agonist caused co-clustering (co-aggregation) of A_1Rs and D_1Rs, which was blocked by combined pretreatment with A_1R and D_1R agonists [69]. These results indicated that the movement of the heteromer and/or clusters of heteromers in the plasma membrane could be agonist-dependent, and that receptor trafficking may depend on the activity of the two receptors of the heteromer. As a result, it has been suggested that D_1R desensitization may be mainly caused by a prolonged allosteric change in the D_1R brought about by the A_1R/D_1R heteromeric complex; specifically these changes could consist of phosphorylation events and/or association with beta-arrestin-like molecules that finally would lead to a reduced Gs coupling of the D_1R [76,77].

Consequently, the A_1R/D_1R receptor–receptor interaction in this heteromer is relevant not only for acute A_1R antagonism of D_1R signaling but also for a persistent long-term antagonism of A_1R to D_1R signaling to the Gs protein. Therefore, it has been postulated that novel therapeutic approaches, for instance in Parkinson's disease, may be developed taking into account not only the particular characteristics of the receptors within the heteromer, but also the fact that the pathology may induce changes in such receptor–receptor interactions involved in the function of neuronal networks of the brain, such as in the basal ganglia and prefrontal cortex [78].

3.2. A_1R and metabotropic glutamate type 1 ($mGlu_1$) receptor oligomers

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and exerts its effects through specific cell surface receptors, namely ionotropic glutamate (iGlu) receptors – that form ion channels – and metabotropic glutamate (mGlu) receptors, which are coupled to G proteins [79,80]. To date, eight members of the metabotropic glutamate receptor family have been identified and categorized into three groups (I, II and III), based on their sequence homology, agonist selectivity, and signal transduction pathways [79]. Group I contains the $mGlu_1$ and $mGlu_5$ receptor subtypes, which are coupled to phospholipase C in transfected cells and have quisqualic acid as their most potent agonist. Five splice variants of the $mGlu_1$ receptor have been described [79,81]. Interestingly, these splice variants differ between them in the length of their C-terminal tail, which might play a role in the subcellular

targeting of the receptor [82]. For instance, the C-terminal tail of the metabotropic glutamate type 1α ($mGlu_{1\alpha}$) receptor interacts with tubulin, and it has been proposed that the latter can regulate the cell surface expression of the receptor and its plasma membrane anchoring [83,84]. Noteworthy, another cytoskeletal protein, 4.1 G, has also been described as a binding partner of the $mGlu_{1\alpha}$ receptor [85]. This multifunctional protein, which is a critical component of the spectrin/actin cytoskeleton, plays an important structural and regulatory role in the stabilization and assembly of receptors into the membranes. Interestingly, the 4.1 G protein has also been found to bind to the third intracellular loop of the A_1R and to play a regulatory role in the trafficking and down-regulation of this receptor [85].

As described earlier, among the numerous neurophysiological actions of adenosine, the inhibition of glutamate neurotransmission has been studied in several brain regions [7,86]. The activation of presynaptic adenosine A_1Rs, which leads to a direct inhibitory effect of G protein βγ-subunits on voltage-dependent Ca^{2+} channels, has been described as one of the mechanisms involved in the decrease of the probability of glutamate release to the synaptic space [53,87–89]. Interestingly, the A_1R-mediated fine-tuning modulation of glutamatergic neurotransmission has been demonstrated to occur, at least in part, by means of an interaction between the A_1R and the $mGlu_{1\alpha}$ receptor [35]. Thus, while $A_1R/mGlu_{1\alpha}$ receptor–receptor coupling could occur through specific targeting proteins, such as protein 4.1 G, a direct receptor–receptor interaction between the A_1R and the $mGlu_{1\alpha}$ receptor would also be possible. In fact, the C-terminal tail of the $mGlu_{1\alpha}$ receptor has been shown to be necessary to reach the $A_1R/mGlu_{1\alpha}$ receptor heteromer formation, since other splice variants, as the $mGlu_{1\beta}$ receptor with a shorter C-terminal tail or a deleted mutant of the $mGlu_{1\alpha}$ receptor lacking the C-terminal cytoplasmatic domain did not interact with the adenosine A_1R [35]. In these experiments biochemical and functional evidence was also provided for a $A_1R/mGlu_{1\alpha}$ receptor receptor–receptor interaction. Co-immunoprecipitation experiments showed a close and subtype-specific interaction between A_1Rs and $mGlu_{1\alpha}$ receptors in both rat cerebellar synaptosomes and co-transfected HEK-293 cells. Also, in primary cultures of cortical neurons a high degree of co-localization was also observed [35]. In these rat cortical neurons, it was suggested by means of NMDA-mediated excitotoxicity experiments that the timing in A_1R and $mGlu_{1\alpha}$ receptor activation is very important to achieve a maximum effect in adenosine- and glutamate-mediated neuroprotection/neurodegeneration. Similarly, A_1Rs and $mGlu_{1\alpha}$ receptors may form part of a signalling complex *in vivo* that could play a critical role in fine-tuning neurotransmission at glutamatergic synapses. This was substantiated by using transiently co-transfected HEK-293 cells, in which a synergy between both receptors in receptor-evoked $[Ca^{2+}]_i$ signaling was shown, as previously reported [90]. Overall, these results demonstrate that a functional cross-talk occurs between A_1Rs and $mGlu_{1\alpha}$ receptors. These results open new perspectives for the development of novel agents to treat neuropsychiatric disorders in which abnormal glutamatergic neurotransmission is involved.

4. Adenosine A_2 receptor (A_2R) containing oligomers

4.1. $A_{2A}R$ and dopamine D_2 receptor (D_2R) oligomers

In the striatum, more than the 95% of the neuronal population is comprised by the medium spiny GABAergic efferent neurons. There are two subtypes of these GABAergic striatal neurons, the GABAergic striatopallidal neurons, which can be called enkephalinergic neurons, since they express the peptide enkephalin, and the GABAergic striatonigral-striatoentopeduncular neurons, called also as GABAergic dynorphynergic neurons, since they express the peptide dynorphin (and also substance P). Interestingly, while the GABAergic enkephalinergic neuron predominantly expresses A_{2A}Rs and D_2Rs, the GABAergic dynorphynergic neuron expresses A_1Rs and D_1Rs [18,67,91]. Currently, it is well accepted that the $A_{2A}R$ plays a modulatory role in the CNS in general and in the striatum in particular, and that this $A_{2A}R$-mediated neuromodulatory role is somehow related to the ability of this receptor to heteromerize with other GPCRs. Indeed, in the last decade the existence of a direct receptor–receptor interaction between A_{2A}Rs and D_2Rs has been demonstrated [38,92]. Thus, it has been postulated that while the previously described A_1R/D_1R interaction would modulate the function of the GABAergic dynorphynergic neurons, the $A_{2A}R/D_2R$ interaction would modulate the function of the GABAergic enkephalinergic neurons [67,68].

The $A_{2A}R/D_2R$ receptor–receptor interaction takes place in both the somatodendritic area and in the nerve terminals of the GABAergic enkephalinergic neurons [40,42,43,68]. At this level, the coexistence of two reciprocal antagonistic interactions between these receptors has been studied. First, it has been demonstrated that the stimulation of D_2Rs in the globus pallidus produces a strong counteraction of $A_{2A}R$-mediated GABA release [93]. Similarly, recent studies in animal models of Parkinson's disease have suggested that the pallidal $A_{2A}R/D_2R$ interaction may contribute to the antiparkinsonian effects of the co-administration of $A_{2A}R$ selective antagonists and L-DOPA or D_2R selective agonists. On the other hand, it has been shown that there exists a strong tonic activation of D_2R blocking the ability of the $A_{2A}R$ to signal through the cAMP-PKA pathway. In this way, *in vivo* administration of a D_2R selective antagonist in the rodent striatum produces a significant increase in the expression of c-fos and preproenkephalin genes, which depends on the ability of the D_2R to tonically block $A_{2A}R$ signaling activated by endogenous adenosine [94]. Overall, two reciprocal antagonistic $A_{2A}R/D_2R$ interactions have been described, namely an intermembrane interaction in which the $A_{2A}R$ mediates the inhibition of the D_2R, thus modulating neuronal excitability and neurotransmitter release, and an interaction at the level of adenylyl-cyclase in which the D_2R inhibits $A_{2A}R$-mediated protein phosphorylation and gene expression.

There are two possible mechanisms to explain the apparently incompatible coexistence of two reciprocal antagonistic $A_{2A}R/D_2R$ interactions. Firstly, it has been suggested that there exists a different G-protein coupling depending on the conformation of the D_2R. Accordingly, it has been proposed that the D_2R couples to Gi/o, and therefore negatively to adenylyl-cyclase, when not being part of the $A_{2A}R/D_2R$ heteromer, while it switches to Gq/11-PLC signaling when heteromerization occurs. And secondly, the presence of a third partner has been proposed, namely the $mGlu_5$ receptor, which could interact with the $A_{2A}R$ (see below). Interestingly, it has been shown both in transfected cells and in the striatum that upon co-stimulation of A_{2A}Rs and $mGlu_5$ receptors a synergistic effect is produced on c-fos expression related to changes at the adenyl-cyclase and the MAPK levels [41,95]. In addition, *in vivo* experiments have demonstrated that co-stimulation A_{2A}Rs and $mGlu_5$Rs in the presence of a selective D_2R agonist permits the $A_{2A}R$ to be liberated from the tonic inhibitory effect of the D_2R on A_{2A} signals through the cAMP-PKA pathway [96].

In the last years, we and others have demonstrated the existence of the $A_{2A}R/D_2R$ heteromer in living cells by biochemical (i.e. co-immunoprecipitation) and by biophysical (i.e. FRET and BRET) approaches [38,40,97–99]. In addition, by computerized modeling, and pull-down and mass spectrometry techniques, we have also demonstrated that the heteromerization between the $A_{2A}R$ and the D_2R depends on a coulombic interaction between the third intracellular loop (3IL) of the D_2R and the C-terminal tail of the $A_{2A}R$ receptor [38,92,100,101]. Therefore, a positively charged arginine rich motif located in the N-terminal part of the D_2R-3IL may interact with two different negatively charged motifs from the C-terminal tail of the $A_{2A}R$, namely the ^{388}HELKGVCPEPPGL**DD**PLAQDGAVGS^{412} domain which contains two adjacent aspartic residues or the ^{370}SAQE**pS**QGNT^{378} domain which contains a phosphorylable serine residue (S374), and thus forming electrostatic bonds of covalent-like strength [100,101].

Interestingly, we have recently demonstrated that the point mutation of the serine 374 to alanine (S374A) reduces $A_{2A}R/D_2R$ heteromerization and blocks the $A_{2A}R$ allosteric modulation of the dopamine D_2R [102], a result that has been further confirmed [103]. Also, when the S374A point substitution was accompanied with the mutation of the two negatively charged aspartates in the $A_{2A}R$ C-terminal tail (D401A/D402A, see above) a synergistic reduction in the physical $A_{2A}R/D_2R$ interaction was observed and in the loss of antagonistic allosteric modulation over the $A_{2A}R/D_2R$ interface [104]. Thus, these results further corroborate the existence of an electrostatic interaction between the C-terminal tail of the $A_{2A}R$ and the 3IL of the D_2R. In addition, by using synthetic transmembrane (TM) α-helix peptides of the D_2R we have recently explored the role of TM helix interactions within the $A_{2A}R/D_2R$ heteromer interface [104]. Thus, we have evidence that the TM domains IV and V of the D_2R play a critical role in the $A_{2A}R/D_2R$ heteromer interface since the incubation with peptides corresponding to these domains significantly reduced the ability of the $A_{2A}R$ and the D_2R to heteromerize. Also, the incubation with TM-IV or TM-V blocked the allosteric modulation normally found in the $A_{2A}R/D_2R$ heteromer [104]. Overall, the $A_{2A}R$ double aspartate mutation (D401A/D402A) together with the serine mutation (S374A) had deleterious consequences for the allosteric communication within the $A_{2A}R/D_2R$ heteromer, thus pointing toward the relevance of these residues in the formation of an electrostatic zip between the $A_{2A}R$ and the D_2R, which is regulated by phosphorylation events, and thus necessary for the direct physical receptor–receptor cross-talk.

4.2. $A_{2A}R$ and metabotropic glutamate type 5 ($mGlu_5$) receptor oligomers

In some behavioral animal models the treatment with $mGlu_5$ receptor agonists and antagonists produce similar effects to that described for the treatment with $A_{2A}R$ agonists and antagonists, respectively, namely the selective modulation of D_2R-mediated effects. Briefly, while a selective $mGlu_5$ receptor agonist mainly inhibits the D_2R agonists-mediated motor activation [105], a mGlu5 receptor antagonist counteract the effects of D_2R antagonists [106]. Thus, $A_{2A}R$ and $mGlu_5$ receptor agonists and $A_{2A}R$ and $mGlu_5$ receptor antagonists showed synergistic effects at the behavioral level [105,107,108]. In addition, in the striopallidal complex the $mGlu_5$ receptor showed a very similar localization to that described for the $A_{2A}R$ [52,109]. Also, the $mGlu_5$ receptor immunoreactivity was commonly found perisynaptically to asymmetric postsynaptic synapses [109,110]. Overall, these studies provided behavioral and morphological basis for the possible existence of functional interactions between striatal $A_{2A}Rs$ and $mGlu_5$ receptors.

Interestingly, it has been possible to demonstrate the existence of heteromeric receptor complexes between $A_{2A}Rs$ and $mGlu_5$ receptors in both living cells (i.e. HEK-293 cells) and in native tissue preparations (i.e. rat striatal membranes) [41]. Thus, it was initially found that $A_{2A}Rs$ and $mGlu_5$ receptors were co-distributed at the membrane surface of co-transfected HEK-293 cells and also that they were co-immunoprecipitated from cell extracts when specific antibodies were used [41]. Also, a strong $A_{2A}R$ and $mGlu_5$ receptor co-distribution in rat striatal primary cultures was shown [40]. On the other hand, a number of studies have focused on the functionality of the $A_{2A}R/mGlu_5$ receptor heteromeric complex, and it has been suggested that the former oligomer may play a major role on striatal neuronal function and dysfunction. Accordingly, functional $A_{2A}R/mGlu_5$ receptor synergistic interactions have been demonstrated both in co-transfected cells and in the rat striatum. Thus, in HEK-293 cells it was shown that upon $A_{2A}R/mGlu_5$ receptor co-activation a synergistic interaction occurred at the level of extracellular signal-regulated kinase 1/2 phosphorylation (ERK) and of c-fos expression [41]. Interestingly, this synergism was shown to occur at the level of the mitogen-activated protein kinase (MAPK) cascade [111,112]; alternatively, it could also happen that upon co-activation of the oligomer, this receptor complex would assemble with receptor tyrosine kinases or non-receptor tyrosine kinase Src, leading to ERK activation [113–117]. Altogether, it has been then suggested that the $A_{2A}R/mGlu_5$ receptor heteromeric complex may be involved in striatal neuron plasticity (e.g. long-term potentiation and long-term depression) [111,112]. In this way, it has been suggested the possibility of a synergic interaction within the $A_{2A}R/mGlu_5$ receptor oligomeric complex modulating $mGlu_5$ receptor desensitization, which has been demonstrated for $NMDA/mGlu_5$ receptor and group II mGlu/$mGlu_5$ receptor interactions [118,119]. However, this interaction would be dependent on a synergistic interaction at the second-messenger level (Ca^{2+} mobilization) [118,119], a situation that has not been possible to demonstrate in $A_{2A}R/mGlu_5$ receptor cotransfected HEK-293 cells [41].

Similar to the data obtained in cultured cells, studies in striatal slices have shown that stimulation of the $mGlu_5$ receptor potentiates $A_{2A}R$ signalling in a MAPK-dependent manner [95]. Interestingly, MAPK activation is involved in the recruitment of ionotropic glutamate AMPA receptors to the postsynaptic density [120,121], thus the $A_{2A}R/mGlu_5$ receptor heteromer would be able to modulate plastic changes in the striatal spine module (SSM, see below). In fact, it has been demonstrated that the pharmacological or genetic inactivation of $A_{2A}Rs$ or $mGlu_5$ receptors impairs corticostriatal long-term potentiation [122,123]. The $A_{2A}R/mGlu_5$ receptor synergism has also been demonstrated in the rat striatum, mediating c-fos expression and thus counteracting phencyclidine induced motor activity [41]. Interestingly, it is well known that this motor activity is dependent on dopamine D_2R activity, thus it has been suggested, as commented above, that upon $A_{2A}R/mGlu_5$ receptor co-stimulation it is blocked D_2R mediated transmission at the behavioral level. Similarly, chronic but not acute treatment with a $mGlu_5$ receptor antagonist reversed the akinetic deficit in a model of Parkinson's disease [124]. In this case, it would seem that the potentiation of D_2R activity could in part be caused by the internalisation and down-regulation of the $A_{2A}R/mGlu_5$ receptor heteromeric receptor complex, thus removing the D_2R from inhibition of its signalling. In addition, it has also been shown that an acute effect of $mGlu_5$ receptors on dopamine D_2R activity exists, since a $mGlu_5$ receptor antagonist induced counteraction of the blockade with haloperidol of the D_2R, thus observing in rats reduced rigidity and catalepsy [106], effects that may in part be exerted at the network level (e.g. at the level of the subthalamic–nigral glutamate system).

Overall, $A_{2A}R/mGlu_5$ receptor and also $D_2R/A_{2A}R/mGlu_5$ receptor (see below) interactions provide the rationale for the application of $A_{2A}R$ antagonists and the possible application of $mGlu_5$ receptor antagonists in Parkinson's disease [67,106,107,125,126].

5. The striatal spine module as a paradigm of ARs integrative functioning in the brain

The striatum receives the densest dopamine innervation and contains the highest concentration of dopamine receptors in the brain [127,128]. As a consequence, dopamine plays a key role in motor activity and goal-directed behaviors and also in the pathophysiology of diverse disorders, including Parkinson's disease and drug addiction [39]. Moreover, in this brain area, apart of the pivotal role played by dopamine, there are other neurotransmitters/neuromodulators that participate in the proper striatal functioning, for instance glutamate, acetylcholine, GABA, adenosine, etc., Therefore, interactions among striatal neurotransmitters/neuromodulators determines the types of plasticity taking place at the glutamatergic synapses onto striatal neurons, and thus complex interactions between the receptors for these neurotransmitters/neuromodulators are currently established. Interestingly, we have demonstrated the existence of oligomeric complexes that simultaneously contain adenosine, dopamine and glutamate receptors (e.g., $A_{2A}R/D_2R/mGlu_5$ receptor) and that these

aggregates might be relevant for the proper striatal function [129]. Indeed, the recent fluorescence-based approaches on the study of protein-protein interactions [25] have allowed us to demonstrate the existence of higher-order $A_{2A}R/D_2R/mGlu_5$ receptor oligomers or RMs. Initially, by using bimolecular fluorescence complementation (BiFC), we visualized for the first time the occurrence of $D_2R/mGlu_5$ receptor heterodimers in living cells [129]. Furthermore, the combination of BiFC and BRET techniques allowed us to detect the existence of receptor oligomers containing more than two protomers, namely $A_{2A}R/D_2R/mGlu_5$ receptor higher-order oligomers or RMs [129]. Next, by using triple-labeling post-embedding immunogold and detection at electron microscopic level the precise simultaneous distribution of $A_{2A}R$, D_2R and $mGlu_5$ receptor in striatal neurons have been performed. It is important to mention here that these three receptors co-distributed in post-synaptic structures along the extra-synaptic and peri-synaptic plasma membrane of spines, establishing asymmetrical, putative glutamatergic, synapses with axon terminals [129] (Fig. 2, inset panel). Overall, the $A_{2A}R/D_2R/mGlu_5$ receptor oligomeric complex is located adjacent to the glutamatergic synapse of the dendritic spine of the enkephalin MSN, and their cross-talk within the receptor heteromers might help in theory to modulate postsynaptic plastic changes at the glutamatergic synapse [42,43] (Fig. 2).

The $A_{2A}R$ and group I glutamate metabotropic (mGlu) receptor agonists could synergistically reduce affinity of striatal D_2R. Therefore, in membrane preparations from rat striatum, the stimulation of either the $A_{2A}R$ or the $mGlu_5$ receptor produces a decrease in the affinity of the D_2R for agonists and a decrease in D_2R agonist-mediated motor activation [105,125]. In addition, co-stimulation of $A_{2A}R$ and $mGlu_5$ receptor produces a synergistic antagonistic modulation of D_2R ligand binding and function that is significantly stronger than the reduction induced by stimulation of either receptor alone [105]. Thus, co-administration of $A_{2A}R$ and $mGlu_5$ receptor agonists inhibits motor activation induced by D_2R agonists [41,105]. Therefore, it is postulated that the formation of $A_{2A}R/D_2R/mGlu_5$ receptor oligomer is a key step for the correct shape of the SSM as a functional local module in the striatum (Fig. 2). Interestingly, the SSM, which is constituted by the dendritic spine of the MSN, its glutamatergic and dopaminergic terminals and astroglial processes, could be considered the minimum portion that operates as an integrative independent unit in the striatum and it provides an example of the extraordinary degree of computation that takes place in local modules (Fig. 2) [42,43]. Indeed, the formation of glutamate, dopamine and adenosine receptors oligomers in the striatum are behind the SSM performance as a local module, thus the coordinate operation of these oligomers within the SSM is associated with particular elaborated functions in the local module. In the SSM, the $A_{2A}R/D_2R/mGlu_5$ receptor oligomeric complex is located extrasynaptically and adjacent to the glutamatergic synapse of the dendritic spine of enkephalin MSNs, where they are activated by volume transmission and their cross-talk within the complex helps to modulate postsynaptic plastic changes at the glutamatergic synapse. On the other hand, the $A_1R/A_{2A}R$ heteromeric complex is found in glutamatergic terminals and the molecular cross-talk between the two receptors in the heteromer helps to modulate glutamate release (Fig. 2). Overall, the formation of oligomeric complexes in the SSM containing adenosine receptors allows a much more elaborated tuning in the regulation of both presynaptic and postsynaptic neuronal responses in the striatal local circuitry.

6. Concluding remarks

The existence of GPCR oligomers is now broadly accepted and the functional meaning of receptor oligomerization is becoming revealed (http://data.gpcr-okb.org/gpcr-okb/). In the CNS the oligomerization of neurotransmitter/neuromodulator receptors confers functional entities which possess different biochemical characteristics with respect to the individual components of the heteromer. Thus, the signalling mediated by single stimulation of any receptor within the oligomer may be, from a qualitative and/or quantitative point of view, different from the one obtained upon the multiple stimulation of the oligomer, as a consequence in the CNS the oligomer may function as a computational device that modulates information flow between neurons. In this way, the adenosine receptors in general and the oligomers containing these receptors in particular constitute a clear example of integration of different modes of communication (i.e. wiring transmission *vs* volume transmission) mediated by neurotransmitters and neuromodulators (i.e. dopamine, glutamate, acethylcholine and adenosine). Consequently, the challenge now would be to fully characterize these adenosine containing oligomers in native tissue in order to determine how these oligomers impinge into neuronal functioning in both normal and pathological conditions. Regarding this last issue, several concepts should be taken into account to fully understand the role of these oligomers: the differential spatio-temporal expression pattern of each independent receptor within the oligomer, the stoichiometry and relative affinity of these receptors within the RMs, and the functional and molecular cross-talk between the different receptors of a named oligomer. Overall, the knowledge of these issues will be necessary before the design of any adenosine receptor oligomer-based therapeutic strategy.

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